

# GATA-3 suppresses IFN- $\gamma$ promoter activity independently of binding to *cis*-regulatory elements

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Received 14 May 2004; accepted 10 June 2004

Available online 21 June 2004

Edited by Takashi Gojobori

**Abstract** The regulatory mechanism by which GATA-3 suppresses IFN- $\gamma$  gene expression was investigated. A reduction of GATA-3 using RNA interference technology enhanced, whereas overexpression of GATA-3 suppressed IFN- $\gamma$  mRNA expression. IL-4 expression was reciprocally affected by GATA-3. GATA-3-mediated down-regulation of IFN- $\gamma$  was achieved through the inhibition of its promoter/enhancer activity. Two GATA elements located in the *cis*-regulatory elements did not contribute to the suppression of IFN- $\gamma$  promoter activity, even though they behaved as binding sites for GATA-3. The effect of GATA-3 on IFN- $\gamma$  promoter was lost upon removal of the region encompassing –257 to –172. Among several transcription factors putatively interacting with this region, Stat4, which enhanced IFN- $\gamma$  promoter activity, was down-regulated by GATA-3 at gene transcription level. Although GATA-3 has the capacity to interact with the *cis*-regulatory elements, it suppresses IFN- $\gamma$  gene transcription via down-regulation of Stat4.  
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**Keywords:** IFN- $\gamma$ ; GATA-3; Stat4; Th1/Th2

## 1. Introduction

Helper T (Th) cells develop into at least two distinct subsets with different functional capabilities and cytokine profiles [1,2]. Among a variety of key molecules governing Th1/Th2 development [3,4], GATA-3 has been implicated in Th2 commitment [5–8]. GATA-3 is selectively expressed in Th2 but not Th1 cells [5,6,9,10]. Transgenic and retroviral expression of GATA-3 induces a Th2 cytokine profile in Th1 cells [5,6], while dominant-negative GATA-3 down-regulates Th2 cytokines in Th2 clones [9]. Transfection analysis has revealed that GATA-3 enhances the expression of several Th2 cytokine through the

GATA sites present in *cis*-regulatory elements of those cytokines. In addition, overexpression of GATA-3 in developing Th1 cells induces an accessible chromatin configuration at the IL-4 loci which harbor IL-4, IL-13, and IL-5 [11]. These findings suggest that GATA-3 allows the expression of Th2 cytokines by functioning as a transcription factor as well as by modifying the chromatin structure of the IL-4 loci.

GATA-3 not only transactivates Th2 cytokine genes, but also suppresses Th1 cytokine expression. Ectopic expression of GATA-3 leads to the inhibition of IFN- $\gamma$  production under Th1-polarizing conditions [5,7,8]. In contrast, IFN- $\gamma$  production in CD4<sup>+</sup> T cells of GATA-3-deficient mouse was increased even under Th2 condition [12]. It has been demonstrated that the expression of Th1-accelerating molecules, IL-12R $\beta$ 2 chain and Stat4, in murine T cells were decreased upon retroviral introduction of GATA-3 during cellular differentiation [7,8]. However, it is not known whether the GATA-3-mediated inhibition of IFN- $\gamma$  production is due to a direct effect on its promoter activation pathway or is indirectly regulated by suppression of the Th1 developmental process.

In the present study, we evaluated the role of GATA-3 in IFN- $\gamma$  gene transcription, separately from its overall effects on T helper subset differentiation, by employing a human T cell line, Jurkat Tag cells, that express both Th1 and Th2 cytokines upon stimulation. Recently, it was appreciated that small double-stranded interfering RNAs (siRNA) can be powerful sequence-specific catalysts for targeted RNA destruction by means of an evolutionarily conserved mechanism known as RNA interference [13,14]. Development of an siRNA expression system in mammalian cells [15,16] enabled us, in this study, to analyze the role of GATA-3 in human T cells.

## 2. Materials and methods

### 2.1. Plasmids

Human GATA-3 cDNA was amplified from total cDNA of human peripheral blood lymphocytes using a sense-(5'-CCCACCGAAAGCAAATCATTCACG) and antisense-(5'-TGTGAGCATCGAGCAGGGCTCTAAC) PCR primer pair, and cloned into the pEF6/His expression vector (Invitrogen Corp. Carlsbad, CA, USA). To construct a short hairpin form siRNA (shRNA)-expression vector, sense and antisense 21-bp siRNA sequence derived from the 3'-UTR region of human GATA-3 cDNA (sense; 5'-GTTCTGGGCAATCAGT GTTAC), combined with the loop sequence (5'-ATTACATCAAG GG) were inserted into the pUC19 vector containing the human U6

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**Abbreviations:** EMSA, electrophoresis mobility shift assay; MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate; shRNA, short hairpin form siRNA; siRNA, small double stranded interfering RNA

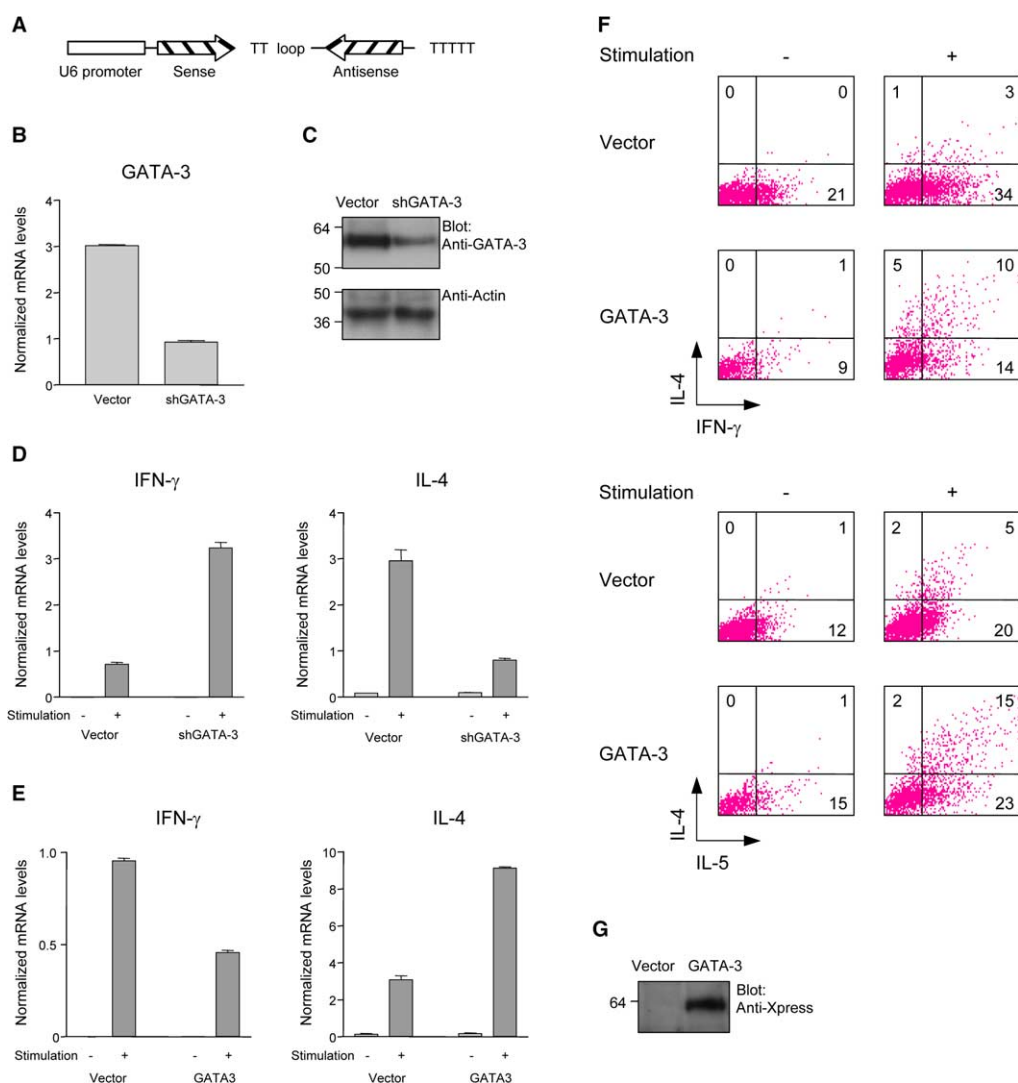


Fig. 1. Effect of GATA-3 shRNA on IFN- $\gamma$  and IL-4 gene expression. (A) Schematic representation of shGATA-3 expression constructs. (B)–(D) Jurkat Tag cells were transfected with empty pU6i vector or shGATA-3 (20  $\mu$ g each) twice on day 0 and 2. (B) and (C) On day 4, the expression of GATA-3 mRNA was analyzed by real-time quantitative PCR (B) and immunoblotting of whole cell lysates with anti-GATA-3 Ab and anti-actin Ab (C). (D) Cells ( $2 \times 10^6$ /ml) were either left unstimulated or stimulated with 25 nM PMA + 1  $\mu$ M ionomycin for 6 h. The expression of IFN- $\gamma$  and IL-4 mRNA was measured by real-time quantitative PCR. Data are expressed as mRNA abundance normalized to GAPDH expression. (E)–(G) Cells were transfected with empty pEF vector, or GATA-3 (10  $\mu$ g each). (E) After 48 h, cells ( $2 \times 10^6$ /ml) were stimulated and the expression of IFN- $\gamma$  and IL-4 was measured as above. (F) In addition to the introduction of empty vector or GATA-3, cells were co-transfected with IFN- $\gamma$ -EGFP or IL-5-EGFP plus IL-4-DsRed-2 reporter plasmids (2  $\mu$ g each). After 48 h, cells ( $10^6$ /ml) were either left unstimulated or stimulated with 25 nM PMA + 1  $\mu$ M ionomycin for 16 h. The promoter activity was determined as the fluorescence of synthetic EGFP and DsRed-2 measured by flow cytometry. (G) The expression of GATA-3 was analyzed by immunoblotting of whole cell lysates with anti-Xpress Ab.

promoter [17] (shGATA-3; Fig. 1A). No matching sequence other than the target *GATA-3* gene was identified as a consequence of BLAST search of the NCBI DNA database. As the reporter constructs, a varying length of the 5'-flanking region of the human *IFN- $\gamma$*  gene (–347 to +2, –257 to +2 and –171 to +2 relative to the transcription initiation site), and the promoter region of the human *IL-5* (–511 to +4) and *Stat4* (–910 to +49) genes were cloned into the pEGFP-1 vector (BD Bioscience Clontech, Palo Alto, CA, USA). The human IL-4 promoter (–1105 to +4) was cloned into the pDsRed-2 vector (BD Bioscience Clontech). The correct sequences of all constructs were verified by sequencing. pRK5-Stat1 and -Stat4 have been described [18].

## 2.2. Measurement of mRNA expression in human T cells

Jurkat Tag cells were cultured and transfected as described [19]. To introduce shRNA-expression vector, the same transfection was repeated after a 48 h interval. At 48 h after final transfection, cells were

treated with phorbol 12-myristate 13-acetate (PMA) (25 nM) plus ionomycin (1  $\mu$ M) for 6 h at 37 °C. Total RNA was then extracted and reverse transcribed using oligo(dT)12–18 primer and ReverTra ACE<sup>®</sup> (Toyobo, Osaka, Japan). Quantitative real-time RT-PCR was performed using Assays-on-Demand<sup>™</sup> Gene Expression Products (TaqMan<sup>®</sup> MGB probes) with an ABI prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). Transcripts of IFN- $\gamma$ , IL-4, GATA-3, Stat1 and Stat4 were normalized to GAPDH abundance.

## 2.3. Reporter assay

After 16 h of stimulation, fluorescence of synthetic EGFP and DsRed-2 in the transfected cells was measured by flow-cytometry. Quantitative data are expressed as mean fluorescence intensity normalized to the fluorescence of DsRed-Express derived from co-transfected pCMV-DsRed-Express vector (BD Bioscience Clontech).

#### 2.4. Electrophoresis mobility shift assay and immunoblotting

Preparation of nuclear and cytoplasmic extracts and electrophoresis mobility shift assay (EMSA) were performed as described [19] using a double-stranded oligonucleotide corresponding to the –115 to –83 site of the human *IFN-γ* gene. For the supershift assay, two anti-GATA-3 Abs (HG3-31 and HG3-35; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were used. Immunoblotting against expressed protein in whole cell lysates was performed using anti-Xpress Ab (Invitrogen), anti-actin Ab, anti-Stat1 Ab and anti-Stat4 Ab (Santa Cruz Biotechnology) as described [19].

### 3. Results and discussion

The first experiment was carried out to investigate the role of GATA-3 in the synthesis of Th1-specific cytokines by reducing endogenously expressed GATA-3 protein. For this purpose, a plasmid carrying shRNA against GATA-3 driven by a human U6 promoter (shGATA-3; Fig. 1A) was transfected into Jurkat Tag cells. As shown in Fig. 1B and C, GATA-3 mRNA and protein were constitutively expressed in Jurkat Tag cells. Introduction of shGATA-3 substantially down-regulated the expression of GATA-3 at the levels of mRNA (Fig. 1B) as well as protein (Fig. 1C). Jurkat Tag cells significantly transcribed both the *IFN-γ* and *IL-4* gene upon stimulation (Fig. 1D). According to the down-regulation of GATA-3 by shGATA-3, the inducible expression of *IFN-γ* was enhanced, while that of *IL-4* was reduced, suggesting that *IFN-γ* expression in human T cells was down-regulated by endogenous GATA-3. The possibility of non-specific and toxic effects of shRNA is most likely to be excluded from these results, because the expression of actin was not affected by shGATA-3 (Fig. 1C). In addition, we confirmed in a separate experiment that shRNA against firefly luciferase gene failed to affect any parameter examined (data not shown). Consistent with the results obtained by shGATA-3, the expression of *IFN-γ* was suppressed, whereas that of *IL-4* was enhanced by ectopic expression of GATA-3 (Fig. 1E). These findings are consistent with other reports showing that *IFN-γ*-producing capacity was decreased and increased in GATA-3-overexpressing and -deficient murine T cells, respectively [7,8,12,20], and further suggest that the down-regulation of *IFN-γ* by GATA-3 was achieved at mRNA expression level.

To clarify, whether GATA-3-mediated suppression of *IFN-γ* is due to the inhibition of gene transcription, the effect of GATA-3 on promoter/enhancer activity of the human *IFN-γ* gene was next analyzed using an improved reporter assay system. The fragment encompassing –347 to +2, which has been reported to display full promoter activity of the human *IFN-γ* gene [21] was coupled to the EGFP reporter gene (*IFN-γ*-EGFP). Human *IL-4* promoter was connected to the DsRed-2 reporter gene (*IL-4*-DsRed-2). These reporters were transfected to Jurkat Tag cells and promoter activity of both Th1 and Th2 specific cytokines were simultaneously measured by flow cytometry. The *IL-5* promoter was also coupled with EGFP (*IL-5*-EGFP). Both *IFN-γ* and *IL-4* promoters were activated upon stimulation (Fig. 1F; top 2 panels). The inducible promoter activity of *IFN-γ* was suppressed, whereas that of *IL-4* was enhanced by overexpression of GATA-3 (Fig. 1F; second panels from top). In *IL-4*-DsRed-2/*IL-5*-EGFP-transfected cells, both inducible *IL-4* and *IL-5* promoter activity were augmented by GATA-3 (Fig. 1F; bottom four panels). The transfected GATA-3 protein was properly expressed in the cells (Fig. 1G). These findings suggest that

GATA-3 specifically down-regulates promoter activity of the human *IFN-γ* gene.

Nevertheless, it has been documented that promoter activity of the murine *IFN-γ* gene was enhanced by GATA-3 [22]. The reason for the apparent contradiction is not clear, but it may be due to the difference in DNA sequence between the murine and human *IFN-γ* promoter regions, which display only 72% identity between positions –300 and –1. Two consensus GATA motifs are present in the regulatory element (–108 to –91-bp) of the human *IFN-γ* gene, with a 9-bp interval (Fig. 2A), though the distal part of the tandem GATA site was not conserved in the murine promoter. In order to clarify, whether and how binding of GATA-3 to the tandem GATA motifs affect *IFN-γ* promoter activity, three mutant reporter constructs were employed. Distal, proximal and both GATA motifs of *IFN-γ*-EGFP were mutated in –347/+2-d-mut, –347/+2-p-mut and –347/+2-dp-mut, respectively (Fig. 2A; bottom three constructs). Binding activity of GATA-3 to these mutant promoters was analyzed by EMSA using an oligonucleotide containing the tandem GATA motifs (–115/–83). At least one specific binding complex was detected with the –115/–83 probe (Fig. 2B). This complex formation was specifically inhibited by the addition of an excess amount of the unlabeled self oligonucleotide and consensus GATA-3 sites in the *IL-5* promoter [10] or the TCRα-enhancer [23] (Fig. 2B; arrow). Furthermore, two specific antibodies against GATA-3 (HG3-31 and HG3-35) formed a supershift complex with this probe, indicating that this specific complex was composed of GATA-3. The –115/–83-d-mut oligonucleotide harboring a mutation in the distal GATA motif inhibited the complex formation less effectively. Further, no reduction of the specific complex formation was achieved by –115/–83-p-mut carrying a mutation in the proximal GATA motif (Fig. 2A and B). A weak and no specific complex was detected with distal and proximal GATA motif-mutated –115/–83 oligonucleotide as probes, respectively (data not shown), suggesting that both GATA motifs contribute to the specific GATA-3 binding and that introduction of a mutation in these motifs, especially the proximal motif, diminishes the binding of GATA-3 to the *cis*-regulatory element of the *IFN-γ* gene.

To determine the functional contribution of the interaction between GATA-3 and the tandem GATA motifs to *IFN-γ* promoter activation, *IFN-γ*-EGFP reporter constructs containing a mutation in the GATA motifs were transfected into Jurkat Tag cells. The inducible *IFN-γ* promoter activity was weakly reduced, if at all, by a substitution mutation of one or both of the GATA motifs (Fig. 2C), suggesting that the binding of GATA-3 to the tandem GATA motifs does not participate in the suppressive effect of GATA-3 on the promoter activity of the human *IFN-γ* gene.

This situation drove us to identify *cis*-regulatory elements contributing to GATA-3-mediated *IFN-γ* promoter down-regulation. Therefore, we next prepared serial 5' deletion constructs of the human *IFN-γ* promoter reporter plasmids (–347/+2). Deletions up to 257-bp from the transcription initiation site (–257/+2 construct) did not affect the suppressive effect of GATA-3 (Fig. 2D). However, the inhibitory activity of GATA-3 was lost upon removal of an 86-bp fragment of the –257/+2 construct (–171/+2). These findings indicate that the *cis*-element located between –257 and –172 is necessary for GATA-3-mediated suppression of *IFN-γ* promoter

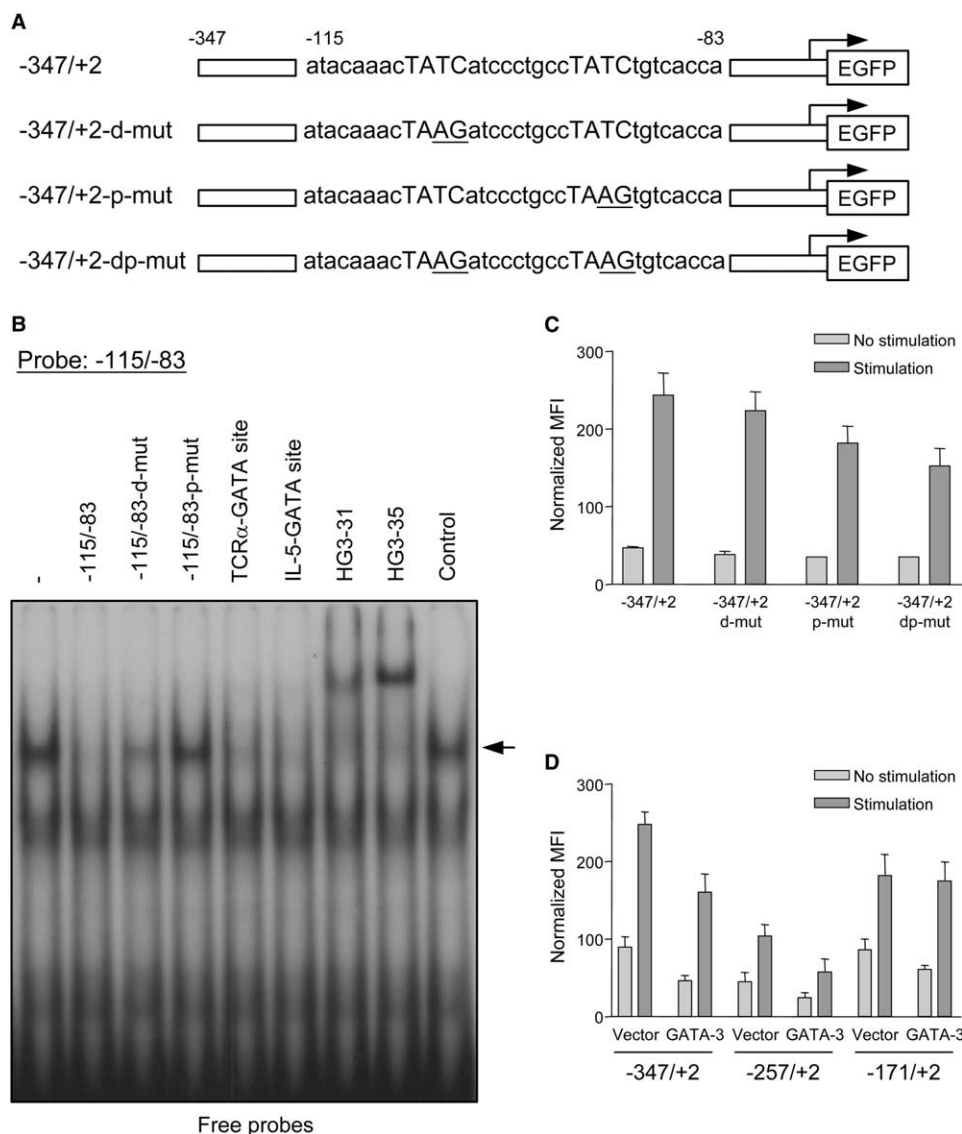


Fig. 2. Contribution of tandem GATA sites to IFN- $\gamma$  gene transcription. (A) Schematic representation of IFN- $\gamma$  promoter reporter mutants. Substitution of the GATA sites is indicated by underlines. (B) Nuclear extract of Jurkat Tag cells was analyzed by EMSA using -115/-83 IFN- $\gamma$  oligonucleotide probe in the absence or presence of the indicated unlabeled competing oligonucleotides, antibodies or control IgG. Arrow indicates specific GATA-3 binding. (C) and (D) Cells were transfected with the -347/+2 IFN- $\gamma$  gene promoter or its mutant-driven EGFP reporter vector in the absence (C) or presence (D) of empty pEF vector or GATA-3. After 48 h, the cells were stimulated and the promoter activity was determined as described in Fig. 1.

activity. Remarkably, the inducible activity of the -171/+2 construct was higher than that of -257/+2, suggesting the possible existence of a silencer element in the region between -257 and -172. It is supported by previous reports showing that a transcription factor, YY1, diminished IFN- $\gamma$  gene transcription by interacting with the promoter regions, -221 to -217 and -203 to -199 [24,25].

No potential GATA-3 binding site exists in the *cis*-element between positions -257 and -172, but this region contains binding sites for other transcription factors, such as AP-1 [26], SP-1 [25], Stat1 and Stat4 [26,27] (Fig. 3A). Among them, Stat4 expression in developing murine Th1 cells was reported to be suppressed by retroviral introduction of GATA-3 [8]. Therefore, we analyzed whether GATA-3 modulates the expression of Stat4 in human T cells. As shown in Fig. 3B and C,

the mRNA level of Stat4 as well as Stat1 was clearly increased upon stimulation. The baseline and inducible expression of Stat4 and Stat1 were augmented by the reduction of endogenous GATA-3 using shRNA (Fig. 3B). In contrast, the ectopic expression of GATA-3 suppressed the expression level of both Stat1 and Stat4 (Fig. 3C). The effect of GATA-3 on Stat4 was further confirmed at gene transcription level using a reporter assay. The fragment of the human *Stat4* gene encompassing -910 to +49 was coupled to the EGFP reporter gene and its activity was measured by flow cytometry. Consistent with the results of mRNA expression, promoter activity of the *Stat4* gene was enhanced upon stimulation (Fig. 3D). The baseline and inducible Stat4 promoter activity were suppressed by the overexpression of GATA-3 (Fig. 3D). In these experiments, proper down-regulation and overexpression of GATA-3

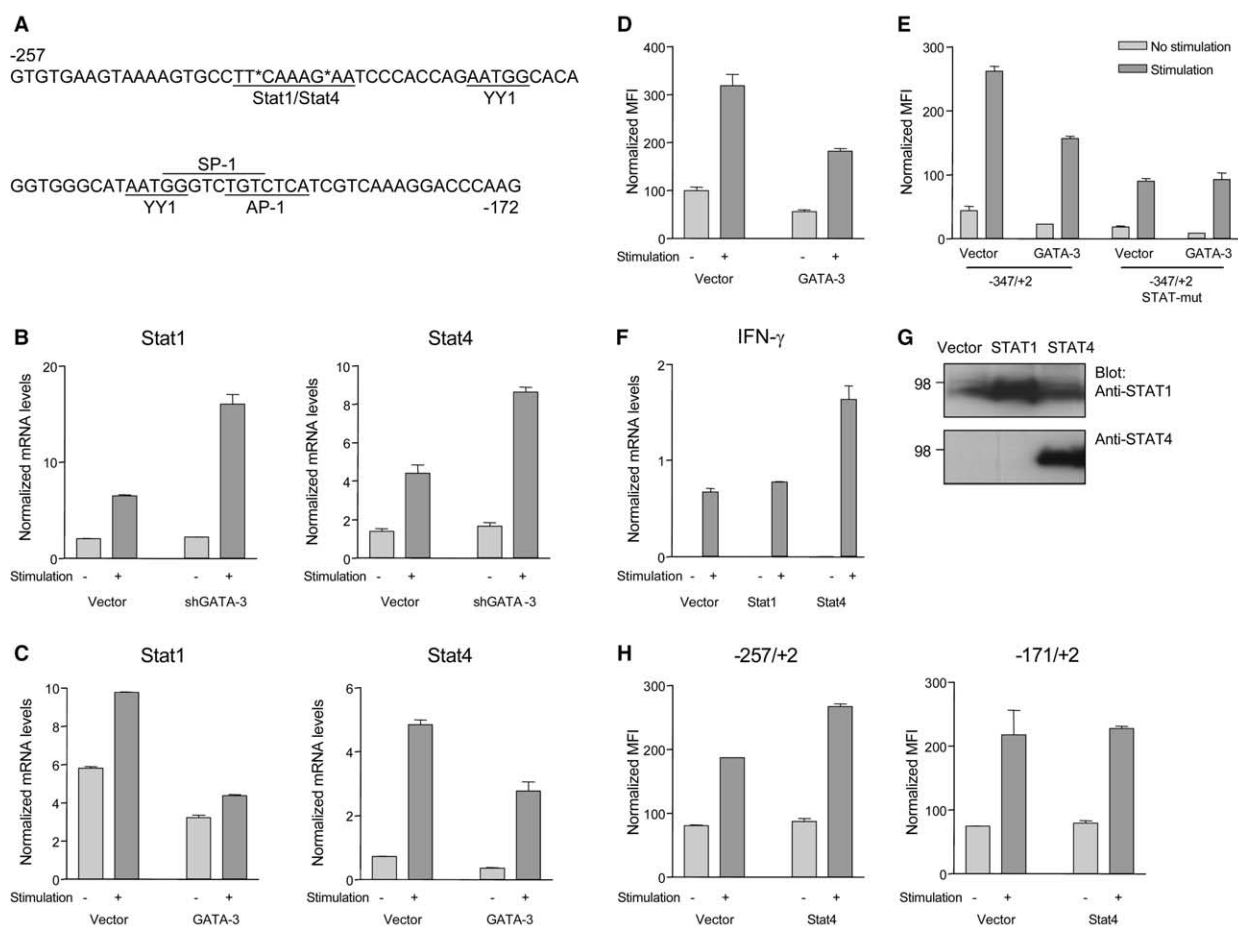


Fig. 3. Role of Stat4 in GATA-3-mediated suppression of IFN- $\gamma$  gene expression. (A) The position of the transcription factor binding sites within -257 to -172 of the human IFN- $\gamma$  gene promoter is shown. Two nucleotides in the Stat1/Stat4 binding site indicated by asterisks were changed in the -347/+2 IFN- $\gamma$ -STAT-mut-EGFP construct. The T was deleted and the G was changed to C. (B) and (C) Cells were transfected with empty pU6i vector or shGATA-3 (B), or with empty pEF vector or GATA-3 (C) and the expression of Stat1 and Stat4 mRNA was measured after stimulation as indicated in Fig. 1. (D) and (E) Cells were co-transfected with Stat4 reporter plasmid (D), -347/+2 IFN- $\gamma$ -EGFP or -347/+2 IFN- $\gamma$ -STAT-mut-EGFP (E) (2  $\mu$ g each) plus empty vector or GATA-3 (10  $\mu$ g each). After 48 h, the cells were stimulated and the promoter activity was determined as in Fig. 1. (F) Cells were transfected with empty pRK5 vector, Stat1 or Stat4 (10  $\mu$ g each). After 48 h, the expression of IFN- $\gamma$  mRNA was measured after stimulation. (G) Whole cell lysates were analyzed for the expression of Stat1 and Stat4 by immunoblotting. (H) Cells were transfected with the -257/+2 or -171/+2 IFN- $\gamma$  gene promoter-driven EGFP vector in the presence of empty pRK5 vector or Stat4. After 48 h, the cells were stimulated and the promoter activity was determined.

protein were obtained as shown in Figs. 1 and 2 (data not shown). These findings are consistent with the previous report regarding murine Th1 cells [8] and further suggest that the down-regulation of Stat4 by GATA-3 in human T cells was achieved at gene transcription level. Many details of the mechanism through which GATA-3 represses Stat4 promoter activity remain to be determined.

Stat4 has been implicated in human IFN- $\gamma$  gene transcription, especially as a signaling molecule in the IL-12 pathway [26,28]. Therefore, we evaluated the effect of Stat4 on IFN- $\gamma$  gene expression. Transcriptional activity of a mutant -347/+2 IFN- $\gamma$  promoter reporter which lost the binding activity with Stat4 [27] was lower than that of the wild type construct (Fig. 3E). This mutant also lost the susceptibility to overexpressed GATA-3. Consistent with previous findings, overexpression of Stat4 clearly enhanced inducible IFN- $\gamma$  expression (Fig. 3F). Stat1, which was also down-regulated by GATA-3 (Fig. 3B and C), failed to affect IFN- $\gamma$  gene expression, even though it was properly overexpressed (Fig. 3F and G). To

confirm that Stat4 up-regulates IFN- $\gamma$  gene expression through its binding site, the effect of Stat4 on the activity of two IFN- $\gamma$  promoter reporter plasmids was examined. As expected, inducible activity of the -256/+2 IFN- $\gamma$ -EGFP construct, containing the Stat sites, was enhanced by overexpression of Stat4 (Fig. 3H). In contrast, activity of the -171/+2 construct, in which the Stat element was truncated, was not affected by Stat4. These findings further support the notion that GATA-3 inhibits IFN- $\gamma$  gene transcription via the down-regulation of Stat4 expression.

Another important regulator of IFN- $\gamma$  gene expression is T-bet. However, neither a reduction nor overexpression of GATA-3 affected the expression level of endogenous T-bet (data not shown), suggesting that Stat4 but not T-bet is involved in the GATA-3-mediated down-regulation of the IFN- $\gamma$  gene transcription. T-bet may operate independently of GATA-3 and/or play a more important role in the regulation of chromatin conformation during commitment to the Th1 lineage.

In conclusion, one of the principal mechanisms through which GATA-3 inhibits IFN- $\gamma$  gene transcription is down-regulation of Stat4. Direct binding of GATA-3 to its corresponding element in the IFN- $\gamma$  promoter region does not participate in the suppression of its transcriptional activity.

*Acknowledgements:* This work was supported by a Grant-in-Aid (to M.S.) from the Japan Health Science Foundation.

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